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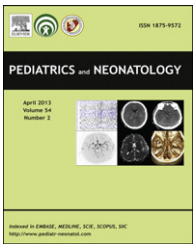

ORIGINAL ARTICLE

Effects of Maternal Retinoic Acid Administration on Lung Angiogenesis in Oligohydramnios-Exposed Fetal Rats

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Key Words
angiogenesis;
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retinoic acid;
vascular endothelial growth factor

Background: All-*trans* retinoic acid (ATRA) induces *in vitro* angiogenesis and vascular endothelial growth factor (VEGF) secretion. Prenatal administration of vitamin A tends to increase the pulmonary and plasma levels of VEGF in the developing mouse. The aims of this study were to examine the effects of maternal retinoic acid treatment on lung VEGF expression and angiogenesis in oligohydramnios-exposed fetal rats.

Methods: On day 16 of gestation, pregnant Sprague–Dawley rats were randomly assigned to either the retinoic acid group (intragastric ATRA at 10 mg/kg body weight) or the vehicle group. We punctured each uterine sac to produce oligohydramnios, and fetuses in the opposite uterine horn served as controls. On day 21 of gestation, the fetuses were delivered by cesarean section.

Results: Rats exposed to oligohydramnios exhibited lower lung weights and lung/body weight ratios, and ATRA exhibited no effects on the body or lung weights of oligohydramnios-exposed rats. Lung microvessel density decreased in oligohydramnios-exposed rats of maternal vehicle-treated dams. Microvessel density was comparable between the oligohydramnios + retinoic acid group and the control + retinoic acid group. VEGF expression was comparable among control and oligohydramnios-exposed rats of maternal vehicle- or retinoic acid-treated dams.

Conclusion: Maternal retinoic acid treatment did not increase lung VEGF expression or enhance lung development in oligohydramnios-exposed fetal rats. These results do not support

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the use of maternal retinoic acid to prevent oligohydramnios-induced pulmonary hypoplasia in the pseudoglandular stage.

1. Introduction

Oligohydramnios may retard fetal lung growth and can result in pulmonary hypoplasia in experimental animals and in human fetuses with prolonged rupture of the membrane.[1,2] The fluid maintains the lungs in an expanded state and provides the tissue with the mechanical stretching necessary for normal lung development.[3] We found that experimental oligohydramnios decreases platelet-derived growth factor expression and epithelial tubules and saccules counts in hypoplastic fetal rat lungs.[4] Vascular endothelial growth factor (VEGF) is a potent endothelial cell mitogen that regulates endothelial cell differentiation and angiogenesis.[5,6] Angiogenesis is physiologically important for alveolarization during normal lung development.[7] The effect of oligohydramnios on lung angiogenesis remains unknown. We hypothesized that experimental oligohydramnios induced defective angiogenesis and decreased VEGF expression in hypoplastic fetal rat lungs.

Vitamin A supplementation is anticipated to improve alveolar formation through its effects on expression of growth factors in the lung that participate in alveolar capillary growth. The rationale for vitamin A supplementation is that vitamin A and its downstream metabolites, such as all-*trans* retinoic acid (ATRA), increase VEGF and VEGF-receptor expression in the lung and maintain alveolar development in mice treated with an angiogenesis inhibitor.[8–10] The aims of this study were to examine the effects of maternal retinoic acid treatment on lung VEGF expression and angiogenesis in oligohydramnios-exposed fetal rats.

2. Materials and Methods

2.1. Experimental design and animal model

This study was approved by the Animal Care and Use Committees of Taipei Medical University and was performed using timed pregnant Sprague–Dawley rats. Five pregnant dams were used for this study. Pregnant dams were randomly assigned to either the retinoic acid group ($n = 3$) or the vehicle group ($n = 2$) on gestational day 16. Each pregnant dam in the retinoic acid group or vehicle group was given a single dose of 3 mL ATRA (10 mg/kg body weight; Sigma, St. Louis, MO, USA) or an equal volume of the vehicle solution (ethanol/olive oil/water in 1:1:1 by volume) by an intragastric route without anesthesia. ATRA was administered on day 16 of gestation to precede the period when the fetal rat lung retinyl palmitate concentration rapidly increases to a peak on gestational day 17.[11] The dosage was based on a study by Shenai and Chytil,[12] who reported that a single dose of retinyl palmitate by maternal intragastric administration on gestational day 16 led to a significant increase in fetal lung retinyl esters within 24 h, and that the effect persisted until postnatal day 14. The dams were anesthetized with pentobarbital (50 mg/kg, i.p.) 1 hour after intragastric retinoic acid or vehicle administration. An abdominal midline incision was made, and the two uterine horns were exposed and kept moist with normal saline. The uterine wall and fetal membranes of each uterine sac in one horn were punctured using a 19-gauge needle, which resulted in an immediate visible leakage of amniotic fluid and a decrease in the size of the uterine sac. Fetuses in the opposite uterine horn served as controls. The uterus was returned to the abdomen, and the abdominal incision was repaired in two layers with silk sutures. The dam was placed in an isolated cage and allowed to recover. On day 21 of gestation, the dams were anesthetized with pentobarbital (50 mg/kg, i.p.), and the fetuses were delivered by cesarean section. The fetuses were weighed and killed by pentobarbital (100 mg/kg, i.p.); then, a ventral midline incision was made, the lungs were dissected free and weighed, and results were expressed as the ratio (%) of lung/body weights.

2.2. Morphological analysis

The lungs were removed and fixed in 10% neutral buffered formalin. Lung sections were cut at 4-μm thickness and stained with hematoxylin and eosin to observe the general morphology. Five portions of every section were randomly selected and captured at ×200 magnifications, and then analyzed using the computerized image analysis system (Image-Pro Plus 5.1 for Windows; Media Cybernetics, Inc., Bethesda, MD, USA) to examine the septal thickness.[13] Digitized images from four nonoverlapping fields of each section were captured. Then, images were printed and examined. The number of points that fell on the airspace was counted by superimposing the transparent grids (196 points) onto the enlarged printed images. All points falling on the component were counted from the nonoverlapping fields of view until all sections from each animal were counted. Airspace volume fraction was defined as the number of points falling on airspace divided by the total number of test points.[14]

2.3. Microvessel density and immunohistochemistry of VEGF

The right superior lobe was embedded in paraffin, and 7-μm sections were cut for histochemical analysis. After deparaffinization in xylene and rehydration in an alcohol series, the sections were first preincubated for 1 hour at room temperature in 0.1M phosphate-buffered saline containing 10% normal goat serum and 0.3% H_2O_2 to block endogenous peroxidase activity and nonspecific binding of antibody before being incubated for 20 hours at 4°C with rabbit polyclonal antibody von Willebrand factor (vWF; 1:200,

Dako, Carpinteria, CA, USA) and rabbit polyclonal antibody VEGF (1:200, Santa Cruz Biotechnology, Inc., CA, USA). The sections were then treated for 1 hour at room temperature with biotinylated goat antirabbit IgG (1:200, Vector, CA, USA). This was followed by the reaction with the reagents from an ABC kit (Avidin–Biotin Complex; Vector Laboratories, Burlingame, CA, USA) as per the manufacturer's recommendations. Microvessel density was determined by counting the vessels staining positively for vWF at ×200 magnifications.[15] For VEGF quantification, a minimum of four random lung fields of immunohistochemistry-stained sections per animal at ×200 magnifications were captured with a digital camera and imported into the computerized image analysis system Image-Pro Plus.[16] The automatic object counting and measuring process was used to quantify the immunoreactivity in the sections. This generated a percentage of positively stained cells and the values were expressed as labeling index (%).

2.4. Statistical analysis

Results are presented as the mean ± SD. The analysis of difference among multiple groups was carried out using one-way analysis of variance, and significance was determined using Bonferroni correction for multiple comparisons. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Body weight, lung weight, and the lung/body weight ratio (%)

The effects of oligohydramnios and maternal retinoic acid treatment on the fetal body weight, lung weight, and lung/body weight ratio (%) are presented in Table 1. Rats exposed to oligohydramnios exhibited significantly lower lung weights and lung/body weight ratios when compared with their control littermates. Maternal retinoic acid treatment had no effects on the body or lung weights of oligohydramnios-exposed rats. Maternal retinoic acid treatment significantly increased the body weight of the control rats compared to control rats of maternal vehicle-treated dams.

3.2. Morphological analysis

The histological appearances of hematoxylin and eosin-stained lungs are illustrated in Figure 1. The representative lung sections exhibit a thicker interstitium and fewer saccules in oligohydramnios-exposed rats in the maternal vehicle- or retinoic acid-treated groups (Figure 1A). Volume fractions of the airspace were significantly lower, and septal thickness was significantly greater in the oligohydramnios + vehicle and oligohydramnios + retinoic acid groups compared with control animals (Figure 1B). Volume fractions and septal thickness of the airspace were comparable between the control + vehicle group and the control + retinoic acid group.

3.3. Microvessel density

To explore angiogenesis in the lung, we performed immunohistochemical staining using an antibody (vWF), a specific marker for endothelial cells. In oligohydramnios-exposed animals, the microvascular density per field of lung tissue tended to be lower compared with that of the control + vehicle group (Figure 2, $p = 0.086$). Microvessel density in the oligohydramnios + retinoic acid group was similar with that in the control + retinoic acid group.

3.4. Immunohistochemistry of VEGF

Immunoreactivities of VEGF were mainly detected in airway epithelial cells and some mesenchymal cells, and the immunoreactivity was comparable among the four groups (Figure 3).

4. Discussion

The effects of maternal retinoic acid treatment on VEGF expression and lung angiogenesis in oligohydramnios-exposed fetal rats are largely unknown. In the present study, the late gestational exposure of fetal rats to oligohydramnios for 5 days decreased fetal lung angiogenesis and had no effect on VEGF expression. Concomitant maternal retinoic acid treatment at a dose of 10 mg/kg did not increase VEGF expression or enhance fetal lung angiogenesis. We have found that maternal retinoic acid treatment increased platelet-derived growth factor-A and -B mRNA expression in oligohydramnios-exposed fetal rat lungs but did not enhance fetal lung development.[17] The present study further suggests that the development of pulmonary vasculature is complex, and that other angiogenic factors are involved in regulating fetal lung angiogenesis.[18]

Literature reports support the use of rodents for studying oligohydramnios because their relative timing of

Table 1 Body weight, lung weight, and the lung/body weight ratio in control and oligohydramnios-exposed rats.

Treatment		Body weight (g)	Lung weight (g)	Lung/body weight (%)
Control + vehicle	7	3.75 ± 0.22	0.11 ± 0.01	3.06 ± 0.20
Oligohydramnios + vehicle	5	3.38 ± 0.35	0.08 ± 0.02[§]	2.37 ± 0.53[*]
Control + retinoic acid	7	4.06 ± 0.27[†]	0.12 ± 0.01	2.95 ± 0.26
Oligohydramnios + retinoic acid	9	3.78 ± 0.36	0.09 ± 0.01[‡]	2.52 ± 0.36[*]

[*] $p < 0.05$, compared with the control + vehicle group.
[†] $p < 0.01$, compared with the control + vehicle group.
[‡] $p < 0.05$, compared with the control + retinoic acid group.
[§] $p < 0.01$, compared with the control + vehicle and control + retinoic acid groups.

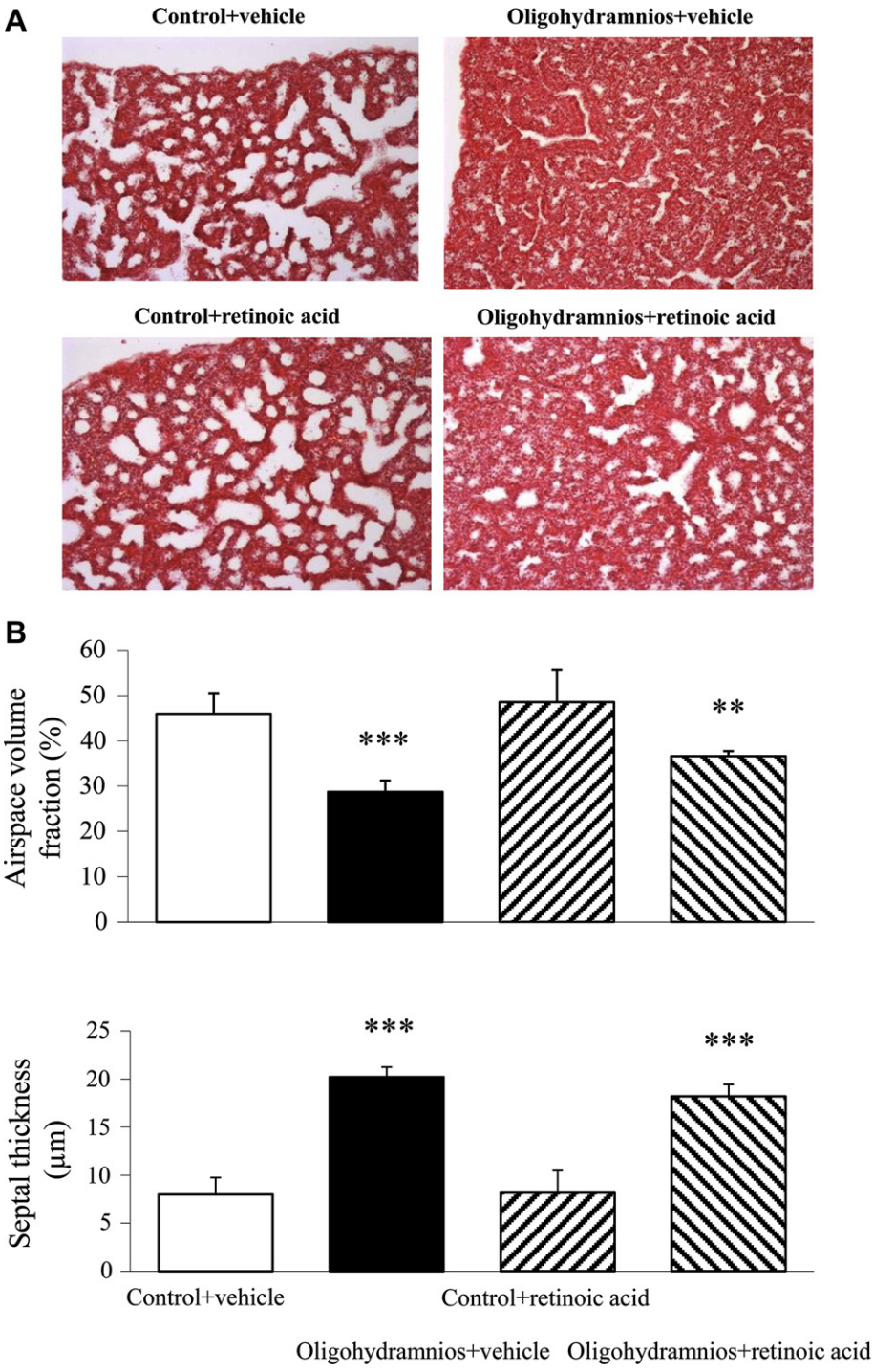

Figure 1 (A) Representative photomicrographs (×200) and (B) volume fractions and septal thickness of the airspace in the lung sections. Oligohydramnios-exposed rats exhibited a thicker interstitium, fewer and less-advanced saccules, and fewer bronchial passageways when compared with control animals. Volume fractions and septal thickness of the airspace were comparable between the control + vehicle group and control + retinoic acid groups. Volume fractions of the airspace were significantly lower and septal thickness was significantly greater in the oligohydramnios + vehicle and oligohydramnios + retinoic acid groups compared with control groups. ***$p < 0.001$, **$p < 0.01$, compared with the control + vehicle and control + retinoic acid groups.

alveolarization resembles human lung development.[19] Oligohydramnios may retard fetal lung growth and result in pulmonary hypoplasia.[2] Pulmonary hypoplasia was defined as underdevelopment of the lungs with a low lung weight for a given body weight. In the present study, we did produce pulmonary hypoplasia based on lung weight and lung/body weight ratio and histological findings of less airway generation.

In this study, maternal retinoic acid treatment had no effects on the lung weights of control rats, but it significantly

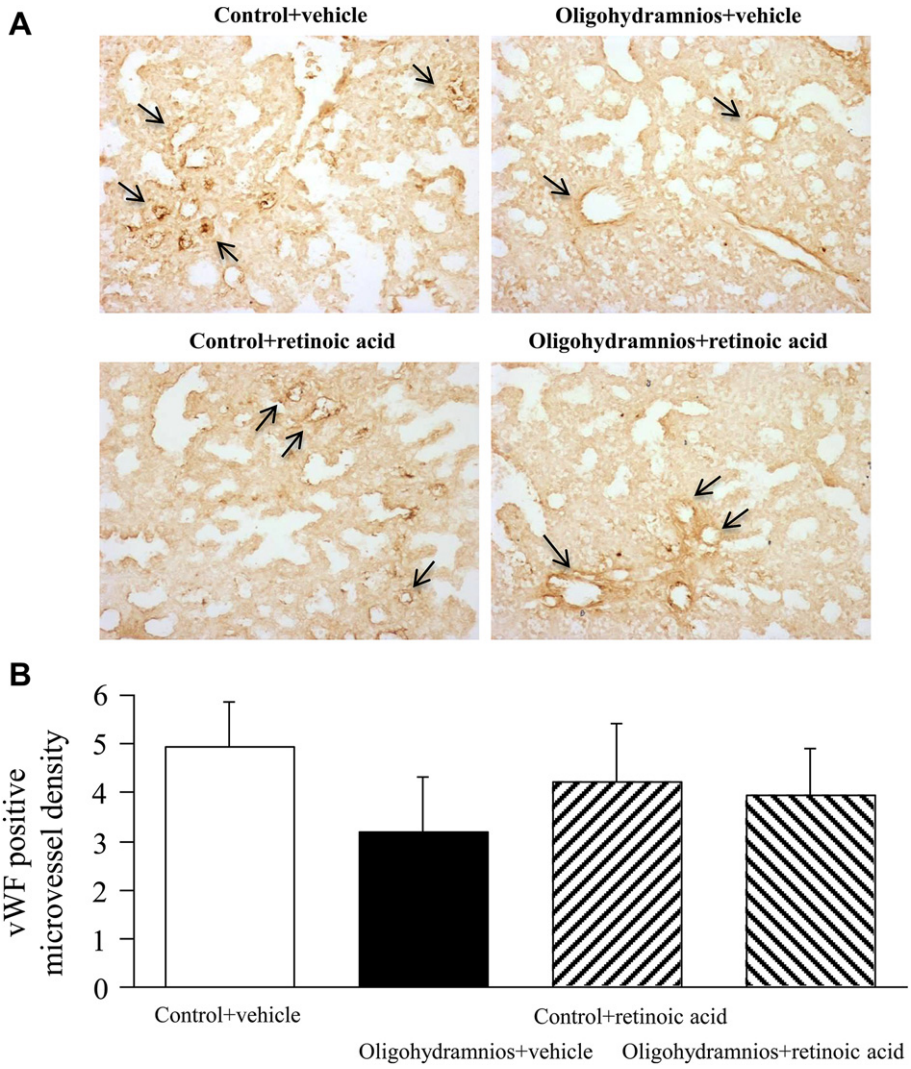

Figure 2 (A) Representative photomicrographs and (B) mean microvessel density in the lung tissues (×200). Microvessel density was determined by counting vessels staining positively for von Willebrand factor. Arrows indicate positively stained microvessels (brown). Microvessel density presented as mean counts per field of view.

increased the body weights of control rats compared to control rats of maternal vehicle-treated dams. These data are consistent with those of Antipatis et al,[20] who demonstrated that a single dose of prenatal retinyl acetate to vitamin A-sufficient pregnant rats on day 10 of gestation increased the body weight of fetuses delivered on day 20 of gestation, and Baptista et al,[21] who found that a late antenatal administration of vitamin A on day 18.5 of gestation did not affect the lung growth of fetuses delivered on day 22 of gestation.

Pulmonary vascular development is a complex biological process that includes vasculogenesis, the *de novo* organization of blood vessels by differentiation of endothelial cells from mesoderm, and angiogenesis, the budding and branching of new vessels from preexisting vessels.[22] This complex and highly organized process requires a delicate orchestration of the regulatory activity of multiple growth factors in a specific temporospatial order. Recent studies suggest that inadequate pulmonary vascular development may lead to underdeveloped airways and gas exchange units.[7,23] Defective angiogenesis has been recognized in hypoplastic lungs in human and in animal models of congenital diaphragmatic hernia.[24] However, lung angiogenesis has rarely been reported in oligohydramnios-associated lung hypoplasia. Microvessel density is a marker of angiogenesis that has been used extensively in the oncologic literature to document the metastatic potential of tumors.[25] VEGF is a potent endothelial cell mitogen that regulates endothelial cell differentiation and angiogenesis.[5,6] VEGF is essential for embryogenesis, as mice deficient in VEGF-A have severe vascular defects that are lethal by embryonic day E8.5 to E9.[26,27] Retinoic acid has been shown to induce VEGF secretion/mRNA expression in human umbilical vein endothelial cells, and prenatal administration of vitamin A tends to increase pulmonary and plasma levels of VEGF in the developing mouse.[8,9] In our rat model, microvessel density tends to be lower in the oligohydramnios-exposed fetal rats, and VEGF protein expression was comparable among control and oligohydramnios-exposed rats of maternal vehicle- or retinoic acid-treated dams.

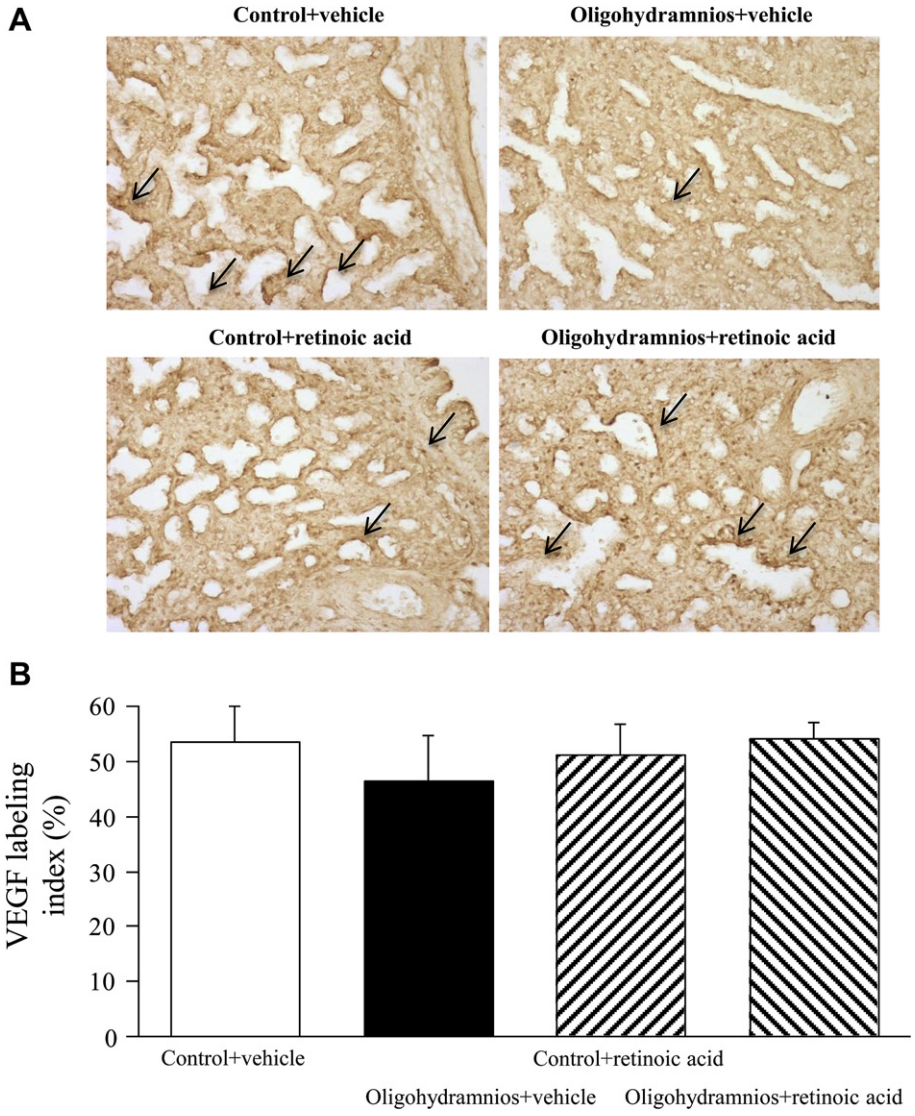

Figure 3 (A) Immunohistochemical staining for vascular endothelial growth factor (VEGF) (×200) and (B) quantitative analysis of VEGF immunoreactivity in the lung sections. Positive staining is shown as brown (arrow). Immunoreactivities of VEGF were mainly detected in airway epithelial and some mesenchymal cells.

ATRA did not increase VEGF expression and failed to stimulate lung angiogenesis as measured by microvessel density. These outcomes are likely to be related to differences in the stage of lung development and the timing and duration of the administration of ATRA.

In conclusion, this study shows that experimental oligohydramnios on day 16 of gestation produces pulmonary hypoplasia and tends to retard angiogenesis on day 21 of gestation in fetal rats. Maternal retinoic acid treatment did not increase lung VEGF expression, and it did not exert positive effects on pulmonary hypoplasia induced by oligohydramnios. This study does not support the use of maternal retinoic acid to prevent or ameliorate oligohydramnios-induced pulmonary hypoplasia in the pseudoglandular stage. Further study is required to determine the effects of the dose and timing of administration of retinoic acid on oligohydramnios-induced pulmonary hypoplasia.

References

1. Thibeault DW, Beatty EC Jr, Hall RT, Bowen SK, O'Neill DH. Neonatal pulmonary hypoplasia with premature rupture of fetal membranes and oligohydramnios. *J Pediatr* 1985;**107**:273—7.
2. Kitterman JA, Chapin CJ, Vanderbilt JN, Porta NF, Scavo LM, Dobbs LG, et al. Effects of oligohydramnios on lung growth and maturation in the fetal rat. *Am J Physiol Lung Cell Mol Physiol* 2002;**282**:L431—9.
3. Alcorn D, Adamson TM, Lambert TF, Maloney JE, Ritchie BC, Robinson PM. Morphological effects of chronic tracheal ligation and drainage in the fetal lamb lung. *J Anat* 1997;**123**:649—60.
4. Chen CM, Wang LF, Chou HC, Lang YD. Oligohydramnios decreases platelet-derived growth factor expression in fetal rat lungs. *Neonatology* 2007;**92**:187—93.
5. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 1989;**246**:1306—9.
6. Peters KG, De Vries C, Williams LT. Vascular endothelial growth factor receptor expression during embryogenesis and tissue

repair suggests a role in endothelial differentiation and blood vessel growth. *Proc Natl Acad Sci U S A* 1993;**90**:8915—9.
7. Jakkula M, Le Cras TD, Gebb S, Hirth KP, Tuder RM, Voelkel NF, et al. Inhibition of angiogenesis decreases alveolization in the developing rat lung. *Am J Physiol Lung Cell Mol Physiol* 2000; **279**:L600—7.
8. Pinto Mde L, Rodrigues P, Coelho AC, Pires Mdos A, dos Santos DL, Gonçalves C, et al. Prenatal administration of vitamin A alters pulmonary and plasma levels of vascular endothelial growth factor in the developing mouse. *Int J Exp Pathol* 2007;**88**:393—401.
9. Saito A, Sugawara A, Uruno A, Kudo M, Kagechika H, Sato Y, et al. All-*trans* retinoic acid induces in vitro angiogenesis via retinoic acid receptor: possible involvement of paracrine effects of endogenous vascular endothelial growth factor signaling. *Endocrinology* 2007;**148**:1412—23.
10. Cho SJ, George CLS, Snyder JM, Acarregui MJ. Retinoic acid and erythropoietin maintain alveolar development in mice treated with an angiogenesis inhibitor. *Am J Respir Cell Mol Biol* 2005; **33**:622—8.
11. Masuyama H, Hiramatsu Y, Kudo T. Effect of retinoids on fetal lung development in the rat. *Biol Neonate* 1995;**67**:264—73.
12. Shenai JP, Chytil F. Effect of maternal vitamin-A administration on fetal lung vitamin-A stores in the perinatal rat. *Biol Neonate* 1990;**58**:318—25.
13. Gao C, Li R, Huan J, Li W. Caveolin-1 siRNA increases the pulmonary microvascular and alveolar epithelial permeability in rats. *J Trauma* 2011;**70**:210—9.
14. Jiang JS, Chou HC, Yeh TF, Chen CM. Maternal nicotine effects on vascular endothelial growth factor expression and morphometry in rat lungs. *Early Hum Dev* 2012;**88**:525—9.
15. Irwin D, Helm K, Campbell N, Imamura M, Fagan K, Harral J, et al. Neonatal lung side population cells demonstrate endothelial potential and are altered in response to hyperoxia-induced lung simplification. *Am J Physiol Lung Cell Mol Physiol* 2007;**293**:L941—51.
16. Chen CM, Chou HC, Wang LF, Lang YD. Captopril decreases plasminogen activator inhibitor-1 in rats with ventilator-induced lung injury. *Crit Care Med* 2008;**36**:1880—5.
17. Chen CM, Chou HC, Wang LF, Lang YD, Yeh CY. Retinoic acid fails to reverse oligohydramnios-induced pulmonary hypoplasia in fetal rats. *Pediatr Res* 2007;**62**:553—8.
18. Stenmark KR, Abman SH. Lung vascular development: implications for the pathogenesis of bronchopulmonary dysplasia. *Annu Rev Physiol* 2005;**67**:623—61.
19. Collins MH, Moessinger AC, Kleinerman J, James LS, Blanc WA. Morphometry of hypoplastic fetal guinea pig lungs following amniotic fluid leak. *Pediatr Res* 1986;**20**:955—60.
20. Antipatis C, Grant G, Ashworth CJ. Moderate maternal vitamin A deficiency affects perinatal organ growth and development in rats. *Br J Nutr* 2000;**84**:125—32.
21. Baptista MJ, Melo-Rocha G, Pedrosa C, Gonzaga S, Teles A, Estevão-Costa J, et al. Antenatal vitamin A administration attenuates lung hypoplasia by interfering with early instead of late determinants of lung underdevelopment in congenital diaphragmatic hernia. *J Pediatr Surg* 2005;**40**:658—65.
22. Baldwin HS. Early embryonic vascular development. *Cardiovasc Res* 1996;**31**:E35—45.
23. Galambos C, Ng YS, Ali A, Noguchi A, Lovejoy S, D'Amore PA, et al. Defective pulmonary development in the absence of heparin-binding vascular endothelial growth factor isoforms. *Am J Respir Cell Mol Biol* 2002;**27**:194—203.
24. Boucherat O, Franco-Montoya ML, Delacourt C, Martinovic J, Masse V, Elie C, et al. Defective angiogenesis in hypoplastic human fetal lungs correlates with nitric oxide synthase deficiency that occurs despite enhanced angiopoietin-2 and VEGF. *Am J Physiol Lung Cell Mol Physiol* 2010;**298**:L849—56.
25. Starnes SL, Duncan BW, Kneebone JM, Fraga CH, States S, Rosenthal GL, et al. Pulmonary microvessel density is a marker of angiogenesis in children after cavopulmonary anastomosis. *J Thorac Cardiovasc Surg* 2000;**120**:902—7.
26. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 1996; **380**:435—9.
27. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 1996;**380**:439—42.